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(54) Title: METHODS FOR GENERATING HYPERMUTABLE YEAST

(57) Abstract: Yeast cells are mutagenized to obtain desirable mutants. Mutagenesis is mediated by a defective mismatch repair system which can be enhanced using conventional exogenously applied mutagens. Yeast cells with the defective mismatch repair system are hypermutable, but after selection of desired mutant yeast strains, they can be rendered genetically stable by restoring the mismatch repair system to proper functionality.

METHODS FOR GENERATING HYPERMUTABLE YEAST

This application claims the benefit of provisional application serial no. 60/184,336 filed February 23, 2000.

FIELD OF THE INVENTION

The invention is related to the area of mismatch repair genes. In particular it is related to the field of *in situ* mutagenesis of single celled organisms.

BACKGROUND OF THE INVENTION

Within the past four years, the genetic cause of the Hereditary Nonpolyposis Colorectal Cancer Syndrome (HNPCC), also known as Lynch syndrome II, has been ascertained for the majority of kindred's affected with the disease (Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. 1996. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. Nat. Med. 2:169-174). The molecular basis of HNPCC involves genetic instability resulting from defective mismatch repair (MMR). To date, six genes have been identified in humans that encode for proteins and appear to participate in the MMR process, including the *mutS* homologs *GTBP, hMSH2,* and *hMSH3* and the *mutL* homologs *hMLH1*, *hPMS1*, and *hPMS2* (Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G., Lescoe, M.K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag,R.J., Godwin, A., R., Ward, D.C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R.M. 1994. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. Nature **368**:258-261; Fishel, R., Lescoe, M., Rao, M.R.S., Copeland, N.J., Jenkins, N.A., Garber, J., Kane, M., and Kolodner, R.

1993. The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. Cell **7**:1027-1038; Leach, F.S., Nicolaides, N.C, Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.Y., Zhang, J., Meltzer, P.S., Yu, J.W., Kao, F.T., Chen, D.J., Cerosaletti, K.M., Fournier, R.E.K., Todd, S., Lewis, T., Leach R.J., Naylor, S.L., Weissenbach, J., Mecklin, J.P., Jarvinen, J.A., Petersen, G.M., Hamilton, S.R., Green, J., Jass, J., Watson, P., Lynch, H.T., Trent, J.M., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. 1993. Mutations of a *mutS* homolog in hereditary non-polyposis colorectal cancer. Cell **75**:1215-1225; Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A., Vogelstein, B., and kinzler, K.W. 1994. Mutations of two PMS homologs in hereditary nonpolyposis colon cancer. Nature **371**: 75-80; Nicolaides, N.C., Palombo, F., Kinzler, K.W., Vogelstein, B., and Jiricny, J. 1996. Molecular cloning of the N-terminus of GTBP. Genomics **31**:395-397; Palombo, F., Hughes, M., Jiricny, J., Truong, O., Hsuan, J. 1994. Mismatch repair and cancer. Nature **36**:417; Palombo, F., Gallinari, P., Iaccarino, I., Lettieri, T., Hughes, M.A., Truong, O., Hsuan, J.J., and Jiricny, J. 1995. GTBP, a 160-kilodalton protein essential for mismatch-binding activity in human cells. Science **268**:1912-1914; Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A.,Vogelstein, B., and Kinzler, K.W. 1994. Mutation of a mutL homolog is associated with hereditary colon cancer. Science **263**:1625-1629). Germline mutations in four of these genes (*hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*) have been identified in HNPCC kindred's (Bronner, C.E., Baker, S.M., Morrison, P.T., Warren, G., Smith, L.G.,

Lescoe, M.K., Kane, M., Earabino, C., Lipford, J., Lindblom, A., Tannergard, P., Bollag,R.J., Godwin, A., R., Ward, D.C., Nordenskjold, M., Fishel, R., Kolodner, R., and Liskay, R.M. 1994. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. Nature **368**:258-261; Leach, F.S., Nicolaides, N.C, Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.Y., Zhang, J., Meltzer, P.S., Yu, J.W., Kao, F.T., Chen, D.J., Cerosaletti, K.M., Fournier, R.E.K., Todd, S., Lewis, T., Leach R.J., Naylor, S.L., Weissenbach, J., Mecklin, J.P., Jarvinen, J.A., Petersen, G.M., Hamilton, S.R., Green, J., Jass, J., Watson, P., Lynch, H.T., Trent, J.M., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. 1993. Mutations of a *mutS* homolog in hereditary non-polyposis colorectal cancer. Cell **75**:1215-1225; Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. 1996. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. Nat. Med. 2:169-174; Nicolaides, N.C., Papadopoulos, N., Liu, B., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A., Vogelstein, B., and kinzler, K.W. 1994. Mutations of two PMS homologs in hereditary nonpolyposis colon cancer. Nature **371**: 75-80; Papadopoulos, N., Nicolaides, N.C., Wei, Y.F., Carter, K.C., Ruben, S.M., Rosen, C.A., Haseltine, W.A., Fleischmann, R.D., Fraser, C.M., Adams, M.D., Venter, C.J., Dunlop, M.G., Hamilton, S.R., Petersen, G.M., de la Chapelle, A.,Vogelstein, B., and kinzler, K.W. 1994. Mutation of a mutL homolog is associated with hereditary colon cancer. Science **263**:1625-1629). Though the mutator defect that arises from the MMR deficiency can affect any DNA sequence, microsatellite sequences are particularly sensitive to

MMR abnormalities (Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. Science **266**:1959-1960). Microsatellite instability (MI) is therefore a useful indicator of defective MMR. In addition to its occurrence in virtually all tumors arising in HNPCC patients, MI is found in a small fraction of sporadic tumors with distinctive molecular and phenotypic properties (Perucho, M. 1996. Cancer of the microsattelite mutator phenotype. Biol Chem. **377**:675-684).

HNPCC is inherited in an autosomal dominant fashion, so that the normal cells of affected family members contain one mutant allele of the relevant MMR gene (inherited from an affected parent) and one wild-type allele (inherited from the unaffected parent). During the early stages of tumor development, however, the wild-type allele is inactivated through a somatic mutation, leaving the cell with no functional MMR gene and resulting in a profound defect in MMR activity. Because a somatic mutation in addition to a germ-line mutation is required to generate defective MMR in the tumor cells, this mechanism is generally referred to as one involving two hits, analogous to the biallelic inactivation of tumor suppressor genes that initiate other hereditary cancers (Leach, F.S., Nicolaides, N.C, Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom-Lahti, M., Guan, X.Y., Zhang, J., Meltzer, P.S., Yu, J.W., Kao, F.T., Chen, D.J., Cerosaletti, K.M., Fournier, R.E.K., Todd, S., Lewis, T., Leach R.J., Naylor, S.L., Weissenbach, J., Mecklin, J.P., Jarvinen, J.A., Petersen, G.M., Hamilton, S.R., Green, J., Jass, J., Watson, P., Lynch, H.T., Trent, J.M., de la Chapelle, A., Kinzler, K.W., and Vogelstein, B. 1993. Mutations of a *mutS* homolog in hereditary non-polyposis colorectal cancer. Cell **75**:1215-1225; Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. 1996. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer

patients. Nat. Med. 2:169-174; Parsons, R., Li, G.M., Longley, M.J., Fang, W.H., Papadopolous, N., Jen, J., de la Chapelle, A., Kinzler, K.W., Vogelstein, B., and Modrich, P. 1993. Hypermutability and mismatch repair deficiency in RER^+ tumor cells. Cell 75:1227-1236). In line with this two-hit mechanism, the non-neoplastic cells of HNPCC patients generally retain near normal levels of MMR activity due to the presence of the wild-type allele.

The ability to alter the signal transduction pathways by manipulation of a gene products function, either by over-expression of the wild type protein or a fragment thereof, or by introduction of mutations into specific protein domains of the protein, the so-called dominant-negative inhibitory mutant, were described over a decade in the yeast system *Saccharomyces cerevisiae* by Herskowitz (Nature 329(6136):219-222, 1987). It has been demonstrated that over-expression of wild type gene products can result in a similar, dominant-negative inhibitory phenotype due most likely to the "saturating-out" of a factor, such as a protein, that is present at low levels and necessary for activity; removal of the protein by binding to a high level of its cognate partner results in the same net effect, leading to inactivation of the protein and the associated signal transduction pathway. Recently, work done by Nicolaides et.al. (Nicolaides NC, Littman SJ, Modrich P, Kinzler KW, Vogelstein B 1998. A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype. Mol Cell Biol 18:1635-1641) has demonstrated the utility of introducing dominant negative inhibitory mismatch repair mutants into mammalian cells to confer global DNA hypermutability. The ability to manipulate the MMR process and therefore increase the mutability of the target host genome at will, in this example a mammalian cell, allows for the generation of innovative cell subtypes or variants of the original wild type cells. These variants can be placed under a specified, desired selective process, the result of which is a novel organism that expresses an altered

biological molecule(s) and has a new trait. The concept of creating and introducing dominant negative alleles of a gene, including the MMR alleles, in bacterial cells has been documented to result in genetically altered prokaryotic mismatch repair genes (Aronshtam A, Marinus MG. 1996. Dominant negative mutator mutations in the mutL gene of Escherichia coli. Nucleic Acids Res 24:2498-2504; Wu TH, Marinus MG. 1994. Dominant negative mutator mutations in the mutS gene of Escherichia coli. J Bacteriol **176**:5393-400; Brosh RM Jr, Matson SW. 1995. Mutations in motif II of Escherichia coli DNA helicase II render the enzyme nonfunctional in both mismatch repair and excision repair with differential effects on the unwinding reaction. J Bacteriol **177**:5612-5621). Furthermore, altered MMR activity has been demonstrated when MMR genes from different species including yeast, mammalian cells, and plants are over-expressed (Fishel, R., Lescoe, M., Rao, M.R.S., Copeland, N.J., Jenkins, N.A., Garber, J., Kane, M., and Kolodner, R. 1993. The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. Cell **7**:1027-1038; Studamire B, Quach T, Alani, E. 1998. Saccharomyces cerevisiae Msh2p and Msh6p ATPase activities are both required during mismatch repair. Mol Cell Biol **18**:7590-7601; Alani E, Sokolsky T, Studamire B, Miret JJ, Lahue RS. 1997. Genetic and biochemical analysis of Msh2p-Msh6p: role of ATP hydrolysis and Msh2p-Msh6p subunit interactions in mismatch base pair recognition. Mol Cell Biol **17**:2436-2447; Lipkin SM, Wang V, Jacoby R, Banerjee-Basu S, Baxevanis AD, Lynch HT, Elliott RM, and Collins FS. 2000. MLH3: a DNA mismatch repair gene associated with mammalian microsatellite instability. Nat. Genet. 24:27-35).

There is a continuing need in the art for methods of genetically manipulating useful strains of yeast to increase their performance characteristics and abilities.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for rendering yeast cells hypermutable.

It is another object of the invention to provide hypermutable yeast cells.

It is a further object of the invention to provide a method of mutating a gene of interest in a yeast.

It is yet another object of the present invention to provide a method to produce yeast that are hypermutable.

It is an object of the invention to provide a method to restore normal mismatch repair activity to hypermutable cells following strain selection.

These and other objects of the invention are provided by one or more of the following embodiments. In one embodiment a method is provided for making a hypermutable yeast. A polynucleotide comprising a dominant negative allele of a mismatch repair gene is introduced into a yeast cell. The cell thus becomes hypermutable.

According to another embodiment a homogeneous composition of cultured, hypermutable yeast cells is provided. The yeast cells comprise a dominant negative allele of a mismatch repair gene.

According to still another embodiment of the invention a method is provided for generating a mutation in a gene of interest. A yeast cell culture comprising the gene of interest and a dominant negative allele of a mismatch repair gene is cultivated. The yeast cell is hypermutable. Cells of the culture are tested to determine whether the gene of interest harbors a mutation.

In yet another embodiment of the invention a method is provided for generating a mutation in a gene of interest. A yeast cell comprising the gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene is grown to create a population of mutated, hypermutable yeast cells. The population of mutated, hypermutable yeast

cells is cultivated under trait selection conditions. Yeast cells which grow under trait selection conditions are tested to determine whether the gene of interest harbors a mutation.

Also provided by the present invention is a method for generating enhanced hypermutable yeast. A yeast cell is exposed to a mutagen. The yeast cell is defective in mismatch repair (MMR) due to the presence of a dominant negative allele of at least one MMR gene. An enhanced rate of mutation of the yeast cell is achieved due to the exposure to the mutagen.

According to still another aspect of the invention a method is provided for generating mismatch repair (MMR)-proficient yeast with new output traits. A yeast cell comprising a gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene is grown to create a population of mutated, hypermutable yeast cells. The population of mutated, hypermutable yeast cells is cultivated under trait selection conditions. The yeast cells which grow under trait selection conditions are tested to determine whether the gene of interest harbors a mutation. Normal mismatch repair activity is restored to the yeast cells.

These and other embodiments of the invention provide the art with methods that can generate enhanced mutability in yeast as well as providing single-celled eukaryotic organisms harboring potentially useful mutations to generate novel output traits for commercial applications.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that hypermutable yeast can be made by altering the activity of endogenous mismatch repair activity of host cells. Dominant negative alleles of mismatch repair genes, when introduced and expressed in yeast, increase the rate of spontaneous mutations by reducing the effectiveness of endogenous mismatch repair-mediated DNA repair activity, thereby rendering the yeast highly

susceptible to genetic alterations, *i.e.*, hypermutable. Hypermutable yeast can then be utilized to screen for mutations in a gene or a set of genes in variant siblings that exhibit an output trait(s) not found in the wild-type cells.

The process of mismatch repair, also called mismatch proofreading, is an evolutionarily highly conserved process that is carried out by protein complexes described in cells as disparate as prokaryotic cells such as bacteria to more complex mammalian cells (Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. Science 266:1959-1960; Parsons, R., Li, G.M., Longley, M., Modrich, P., Liu, B., Berk, T., Hamilton, S.R., Kinzler, K.W., and Vogelstein, B. 1995. Mismatch repair deficiency in phenotypically normal human cells. Science 268:738-740; Perucho, M. 1996. Cancer of the microsattelite mutator phenotype. Biol Chem. 377:675-684). A mismatch repair gene is a gene that encodes one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a mismatch repair complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base that is complementary to the older DNA strand. In this way, cells eliminate many mutations that occur as a result of mistakes in DNA replication, resulting in genetic stability of the sibling cells derived from the parental cell.

Some wild type alleles as well as dominant negative alleles cause a mismatch repair defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a mismatch repair gene is the human gene *hPMS2-134*, which carries a truncation mutation at codon 134 (Parsons, R., Li, G.M., Longley, M., Modrich, P., Liu, B., Berk, T., Hamilton, S.R., Kinzler, K.W., and Vogelstein, B. 1995. Mismatch repair deficiency in phenotypically normal

human cells. Science 268:738-740; Nicolaides NC, Littman SJ, Modrich P, Kinzler KW, Vogelstein B 1998. A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype. Mol Cell Biol 18:1635-1641). The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations, which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any mismatch repair allele, which produces such effect, can be used in this invention, whether it is wild-type or altered, whether it derives from mammalian, yeast, fungal, amphibian, insect, plant, or bacteria. In addition, the use of over-expressed wild type MMR gene alleles from human, mouse, plants, and yeast in bacteria has been shown to cause a dominant negative effect on the bacterial hosts MMR activity (Aronshtam A, Marinus MG. 1996. Dominant negative mutator mutations in the mutL gene of *Escherichia coli.* Nucleic Acids Res 24:2498-2504; Wu TH, Marinus MG. 1994. Dominant negative mutator mutations in the mutS gene of Escherichia coli. J Bacteriol **176**:5393-400; Brosh RM Jr, Matson SW. 1995. Mutations in motif II of Escherichia coli DNA helicase II render the enzyme nonfunctional in both mismatch repair and excision repair with differential effects on the unwinding reaction. J Bacteriol **177**:5612-5621; Lipkin SM, Wang V, Jacoby R, Banerjee-Basu S, Baxevanis AD, Lynch HT, Elliott RM, and Collins FS. 2000. MLH3: a DNA mismatch repair gene associated with mammalian microsatellite instability. Nat Genet **24**:27-35). This suggests that perturbation of the multi-component MMR protein complex can be accomplished by introduction of MMR components from other species into yeast.

Dominant negative alleles of a mismatch repair gene can be obtained from the cells of humans, animals, yeast, bacteria, plants or other organisms. Screening cells for defective mismatch repair activity can identify such alleles. Mismatch repair genes may be mutant or wild type. Yeast host MMR may be mutated or not. The term yeast used in this application comprises any organism from the eukaryotic kingdom, including but not limited to Saccharomyces sp., Pichia sp., Schizosaccharomyces sp., Kluyveromyces sp., and other fungi (Gellissen, G. and Hollenberg, CP. Gene 190(1):87-97, 1997). These organisms can be exposed to chemical mutagens or radiation, for example, and can be screened for defective mismatch repair. Genomic DNA, cDNA, mRNA, or protein from any cell encoding a mismatch repair protein can be analyzed for variations from the wild-type sequence. Dominant negative alleles of a mismatch repair gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other mismatch repair genes (Nicolaides NC, Littman SJ, Modrich P, Kinzler KW, Vogelstein B 1998. A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype. Mol Cell Biol 18:1635-1641). Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable yeast can be evaluated by testing the mismatch repair activity (using methods described in Nicolaides NC, Littman SJ, Modrich P, Kinzler KW, Vogelstein B 1998. A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype. Mol Cell Biol 18:1635-1641) caused by the allele in the presence of one or more wild-type alleles to determine if it is a dominant negative allele.

A yeast that over-expresses a wild type mismatch repair allele or a dominant negative allele of a mismatch repair gene will become hypermutable. This means that the spontaneous mutation rate of such yeast is elevated compared to yeast without such alleles. The degree of elevation

of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal yeast as measured as a function of yeast doubling/hour.

According to one aspect of the invention, a polynucleotide encoding either a wild type or a dominant negative form of a mismatch repair protein is introduced into yeast. The gene can be any dominant negative allele encoding a protein which is part of a mismatch repair complex, for example, *mutS, mutL, mutH, or mutY* homologs of the bacterial, yeast, plant or mammalian genes (Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. Science **266:**1959-1960; Prolla, T.A, Pang, Q., Alani, E., Kolodner, R.A., and Liskay, R.M. 1994. MLH1, PMS1, and MSH2 Interaction during the initiation of DNA mismatch repair in yeast. Science **264:**1091-1093). The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide or polypeptide. The molecule can be introduced into the cell by transformation, electroporation, mating, particle bombardment, or other method described in the literature.

Transformation is used herein as any process whereby a polynucleotide or polypeptide is introduced into a cell. The process of transformation can be carried out in a yeast culture using a suspension of cells. The yeast can be any type classified under the eukayotic kingdom as by international convention.

In general, transformation will be carried out using a suspension of cells but other methods can also be employed as long as a sufficient fraction of the treated cells incorporate the polynucleotide or polypeptide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transformation are well known to those skilled in the art. Available techniques to introduce a polynucleotide or polypeptide into

a yeast cell include but are not limited to electroporation, viral transduction, cell fusion, the use of spheroplasts or chemically competent cells (*e.g.*, calcium chloride), and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transformed with the mismatch repair gene or protein, the cell can be propagated and manipulated in either liquid culture or on a solid agar matrix, such as a petri dish. If the transfected cell is stable, the gene will be expressed at a consistent level for many cell generations, and a stable, hypermutable yeast strain results.

An isolated yeast cell can be obtained from a yeast culture by chemically selecting strains using antibiotic selection of an expression vector. If the yeast cell is derived from a single cell, it is defined as a clone. Techniques for single-cell cloning of microorganisms such as yeast are well known in the art.

A polynucleotide encoding a dominant negative form of a mismatch repair protein can be introduced into the genome of yeast or propagated on an extra-chromosomal plasmid, such as the 2-micron plasmid. Selection of clones harboring a mismatch repair gene expression vector can be accomplished by plating cells on synthetic complete medium lacking the appropriate amino acid or other essential nutrient as described (J. C. Schneider and L. Guarente, Methods in Enzymology 194:373,1991). The yeast can be any species for which suitable techniques are available to produce transgenic microorganisms, such as but not limited to genera including *Saccharomyces, Schizosaccharomyces, Pichia, Hansenula, Kluyveromyces* and others.

Any method for making transgenic yeast known in the art can be used. According to one process of producing a transgenic microorganism, the polynucleotide is introduced into the yeast by one of the methods well known to those in the art. Next, the yeast culture is grown under conditions that select for cells in which the polynucleotide encoding the mismatch

repair gene is either incorporated into the host genome as a stable entity or propagated on a self-replicating extra-chromosomal plasmid, and the protein encoded by the polynucleotide fragment transcribed and subsequently translated into a functional protein within the cell. Once transgenic yeast is engineered to harbor the expression construct, it is then propagated to generate and sustain a culture of transgenic yeast indefinitely.

Once a stable, transgenic yeast cell has been engineered to express a defective mismatch repair (MMR) protein, the yeast can be cultivated to create novel mutations in one or more target gene(s) of interest harbored within the same yeast cell. A gene of interest can be any gene naturally possessed by the yeast or one introduced into the yeast host by standard recombinant DNA techniques. The target gene(s) may be known prior to the selection or unknown. One advantage of employing such transgenic yeast cells to induce mutations in resident or extra-chromosomal genes within the yeast is that it is unnecessary to expose the cells to mutagenic insult, whether it is chemical or radiation, to produce a series of random gene alterations in the target gene(s). This is due to the highly efficient nature and the spectrum of naturally occurring mutations that result as a consequence of the altered mismatch repair process. However, it is possible to increase the spectrum and frequency of mutations by the concomitant use of either chemical and/or radiation together with MMR defective cells. The net effect of the combination treatment is an increase in mutation rate in the genetically altered yeast that are useful for producing new output traits. The rate of the combination treatment is higher than the rate using only the MMR-defective cells or only the mutagen with wild-type MMR cells.

MMR-defective yeast of the invention can be used in genetic screens for the direct selection of variant sub-clones that exhibit new output traits with commercially desirable applications. This permits one to bypass

the tedious and time consuming steps of gene identification, isolation and characterization.

Mutations can be detected by analyzing the internally and/or externally mutagenized yeast for alterations in its genotype and/or phenotype. Genes that produce altered phenotypes in MMR-defective microbial cells can be discerned by any of a variety of molecular techniques well known to those in the art. For example, the yeast genome can be isolated and a library of restriction fragments of the yeast genome can be cloned into a plasmid vector. The library can be introduced into a "normal" cell and the cells exhibiting the novel phenotype screened. A plasmid can be isolated from those normal cells that exhibit the novel phenotype and the gene(s) characterized by DNA sequence analysis. Alternatively, differential messenger RNA screen can be employed utilizing driver and tester RNA (derived from wild type and novel mutant, respectively) followed by cloning the differential transcripts and characterizing them by standard molecular biology methods well known to those skilled in the art. Furthermore, if the mutant sought is encoded by an extra-chromosomal plasmid, then following co-expression of the dominant negative MMR gene and the gene of interest, and following phenotypic selection, the plasmid can be isolated from mutant clones and analyzed by DNA sequence analysis using methods well known to those in the art. Phenotypic screening for output traits in MMR-defective mutants can be by biochemical activity and/or a readily observable phenotype of the altered gene product. A mutant phenotype can also be detected by identifying alterations in electrophoretic mobility, DNA binding in the case of transcription factors, spectroscopic properties such as IR, CD, X-ray crystallography or high field NMR analysis, or other physical or structural characteristics of a protein encoded by a mutant gene. It is also possible to screen for altered novel function of a protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the yeast

associated with the function of the gene of interest, whether the gene is known prior to the selection or unknown.

The screening and selection methods discussed are meant to illustrate the potential means of obtaining novel mutants with commercially valuable output traits, but they are not meant to limit the many possible ways in which screening and selection can be carried out by those of skill in the art.

Plasmid expression vectors that harbor a mismatch repair (MMR) gene insert can be used in combination with a number of commercially available regulatory sequences to control both the temporal and quantitative biochemical expression level of the dominant negative MMR protein. The regulatory sequences can be comprised of a promoter, enhancer or promoter/enhancer combination and can be inserted either upstream or downstream of the MMR gene to control the expression level. The regulatory sequences can be any of those well known to those in the art, including but not limited to the AOX1, GAP, GAL1, GAL10, PHO5, and PGK promoters harbored on high or low copy number extra-chromosomal expression vectors or on constructs that are integrated into the genome via homologous recombination. These types of regulatory systems have been disclosed in scientific publications and are familiar to those skilled in the art.

Once a microorganism with a novel, desired output trait of interest is created, the activity of the aberrant MMR activity is desirably attenuated or eliminated by any means known in the art. These include but are not limited to removing an inducer from the culture medium that is responsible for promoter activation, curing a plasmid from a transformed yeast cell, and addition of chemicals, such as 5-fluoro-orotic acid to "loop-out" the gene of interest.

In the case of an inducibly controlled dominant negative MMR allele, expression of the dominant negative MMR gene will be turned on

(induced) to generate a population of hypermutable yeast cells with new output traits. Expression of the dominant negative MMR allele can be rapidly turned off to reconstitute a genetically stable strain that displays a new output trait of commercial interest. The resulting yeast strain is now useful as a stable strain that can be applied to various commercial applications, depending upon the selection process placed upon it.

In cases where genetically deficient mismatch repair yeast [strains such as but not limited to: M1 (mutS) and in EC2416 (mutS delta umuDC), and mutL or mutY strains] are used to derive new output traits, transgenic constructs can be used that express wild type mismatch repair genes sufficient to complement the genetic defect and therefore restore mismatch repair activity of the host after trait selection [Grzesiuk, E. et.al. (Mutagenesis 13;127-132, 1998); Bridges, B.A., et.al. (EMBO J. 16:3349-3356, 1997); LeClerc, J.E., Science 15:1208-1211, 1996); Jaworski, A. et.al. (Proc. Natl. Acad. Sci USA 92:11019-11023, 1995)]. The resulting yeast is genetically stable and can be employed for various commercial applications.

The use of over-expression of foreign (exogenous, transgenic) mismatch repair genes from human and yeast such as MSH2, MLH1, MLH3, etc. have been previously demonstrated to produce a dominant negative mutator phenotype in yeast hosts (Shcherbakova, P.V., Hall, M.C., Lewis, M.S., Bennett, S.E., Martin, K.J., Bushel, P.R., Afshari, C.A., and Kunkel, T.A. Mol. Cell Biol. 21(3):940-951; Studamire B, Quach T, Alani, E. 1998. Saccharomyces cerevisiae Msh2p and Msh6p ATPase activities are both required during mismatch repair. Mol Cell Biol **18**:7590-7601; Alani E, Sokolsky T, Studamire B, Miret JJ, Lahue RS. 1997. Genetic and biochemical analysis of Msh2p-Msh6p: role of ATP hydrolysis and Msh2p-Msh6p subunit interactions in mismatch base pair recognition.
Mol Cell Biol **17**:2436-2447; Lipkin SM, Wang V, Jacoby R, Banerjee-Basu S, Baxevanis AD, Lynch HT, Elliott RM, and Collins FS. 2000.

MLH3: a DNA mismatch repair gene associated with mammalian microsatellite instability. Nat Genet 24:27-35). In addition, the use of yeast strains expressing prokaryotic dominant negative MMR genes as well as hosts that have genomic defects in endogenous MMR proteins have also been previously shown to result in a dominant negative mutator phenotype (Evans, E., Sugawara, N., Haber, J.E., and Alani, E. Mol. Cell. 5(5):789-799, 2000; Aronshtam A, Marinus MG. 1996. Dominant negative mutator mutations in the mutL gene of Escherichia coli. Nucleic Acids Res 24:2498-2504; Wu TH, Marinus MG. 1994. Dominant negative mutator mutations in the mutS gene of Escherichia coli. J Bacteriol 176:5393-400; Brosh RM Jr, Matson SW. 1995. Mutations in motif II of Escherichia coli DNA helicase II render the enzyme nonfunctional in both mismatch repair and excision repair with differential effects on the unwinding reaction. J Bacteriol 177:5612-5621). However, the findings disclosed here teach the use of MMR genes, including the human PMSR2 gene (Nicolaides, N.C., Carter, K.C., Shell, B.K., Papadopoulos, N., Vogelstein, B., and Kinzler, K.W. 1995. Genomic organization of the human PMS2 gene family. Genomics 30:195-206), the related PMS134 truncated MMR gene (Nicolaides N.C., Kinzler, K.W., and Vogelstein, B. 1995. Analysis of the 5' region of PMS2 reveals heterogenous transcripts and a novel overlapping gene. Genomics 29:329-334), the plant mismatch repair genes (U.S. patent application S.N. 09/749,601) and those genes that are homologous to the 134 N-terminal amino acids of the PMS2 gene to create hypermutable yeast.

DNA mutagens can be used in combination with MMR defective yeast hosts to enhance the hypermutable production of genetic alterations. This further reduces MMR activity and is useful for generation of microorganisms with commercially relevant output traits.

The ability to create hypermutable organisms using dominant negative alleles can be used to generate innovative yeast strains that display

new output features useful for a variety of applications, including but not limited to the manufacturing industry, for the generation of new biochemicals, for detoxifying noxious chemicals, either by-products of manufacturing processes or those used as catalysts, as well as helping in remediation of toxins present in the environment, including but not limited to polychlorobenzenes (PCBs), heavy metals and other environmental hazards. Novel yeast strains can be selected for enhanced activity to either produce increased quantity or quality of a protein or non-protein therapeutic molecule by means of biotransformation. Biotransformation is the enzymatic conversion of one chemical intermediate to the next intermediate or product in a pathway or scheme by a microbe or an extract derived from the microbe. There are many examples of biotransformation in use for the commercial manufacturing of important biological and chemical products, including penicillin G, erythromycin, and clavulanic acid. Organisms that are efficient at conversion of "raw" materials to advanced intermediates and/or final products also can perform biotransformation (Berry, A. Trends Biotechnol. 14(7):250-256). The ability to control DNA hypermutability in host yeast strains using a dominant negative MMR (as described above) allows for the generation of variant subtypes that can be selected for new phenotypes of commercial interest, including but not limited to organisms that are toxin-resistant, have the capacity to degrade a toxin *in situ* or the ability to convert a molecule from an intermediate to either an advanced intermediate or a final product. Other applications using dominant negative MMR genes to produce genetic alteration of yeast hosts for new output traits include but are not limited to recombinant production strains that produce higher quantities of a recombinant polypeptide as well as the use of altered endogenous genes that can transform chemical or catalyze manufacturing downstream processes. A regulatable dominant negative MMR phenotype can be used to produce a yeast strain with a commercially beneficial output trait. Using

this process, single-celled yeast cells expressing a dominant negative MMR can be directly selected for the phenotype of interest. Once a selected yeast with a specified output trait is isolated, the hypermutable activity of the dominant negative MMR allele can be turned-off by several methods well known to those skilled in the art. For example, if the dominant-negative allele is expressed by an inducible promoter system, the inducer can be removed or depleted. Sych systems include but are not limited to promoters such as: lactose inducibleGALi-GAL10 promoter (M. Johnston and R.W. Davis, *Mol. Cell Biol.* 4:1440, 1984); the phosphate inducible PHO5 promoter (A. Miyanohara, A. Toh-e, C. Nosaki, F. Nosaki, F. Hamada, N. Ohtomo, and K. Matsubara. *Proc. Natl. Acad. Sci. U.S.A.* 80:1, 1983); the alcohol dehydrogenase I (ADH) and 3-phosphoglycerate kinase (PGK) promoters, that are considered to be constitutive but can be repressed/de-repressed when yeast cells are grown in non-fermentable carbon sources such as but not limited to lactate (G. Ammerer, *Methods in Enzymology* 194:192, 1991; J. Mellor, M.J. Dobson, N.A. Roberts, M.F. Tuite, J.S. Emtage, S. White, D.A. Lowe, T. Patel, A.J. Kingsman, and S.M. Kingsman, Gene 24:563, 1982); S. Hahn and L. Guarente, Science 240:317, 1988); Alcohol oxidase (AOX) in Pichia pastoris (Tschopp, JF, Brust, PF, Cregg, JM, Stillman, CA, and Gingeras, TR. Nucleic Acids Res. 15(9):3859-76, 1987; and the thiamine repressible expression promoter nmt1 in Schizosaccharomyces pombe (Moreno, MB, Duran, A., and Ribas, JC. Yeast 16(9):861-72, 2000). Yeast cells can be transformed by any means known to those skilled in the art, including chemical transformation with LiCl (Mount, R.C., Jordan, B.E., and Hadfield, C. Methods Mol. Biol. 53:139-145,1996) and electroporation (Thompson, JR, Register, E., Curotto, J., Kurtz, M. and Kelly, R. Yeast 14(6):565-71, 1998). Yeast cells that have been transformed with DNA can be selected for growth by a variety of methods, including but not restricted to selectable markers (URA3; Rose, M., Grisafi, P., and Botstein, D. Gene 29:113,1984; LEU2;

A. Andreadis, Y., Hsu, M., Hermodson, G., Kohlhaw, and P. Schimmel. J. Biol. Chem. 259:8059,1984; ARG4; G. Tschumper and J. Carbon. Gene 10:157, 1980; and HIS3; K. Struhl, D.T. Stinchcomb, S., Scherer, and R.W. Davis Proc. Natl. Acad. Sci. U.S.A. 76:1035,1979) and drugs that inhibit growth of yeast cells (tunicamycin, TUN; S. Hahn, J., Pinkham, R. Wei, R., Miller, and L. Guarente. Mol. Cell Biol. 8:655,1988). Recombinant DNA can be introduced into yeast as described above and the yeast vectors can be harbored within the yeast cell either extra-chromosomally or integrated into a specific locus. Extra-chromosomal based yeast expression vectors can be either high copy based (such as the 2-μm vector Yep13; A.B. Rose and J.R. Broach, Methods in Enzymology 185:234,1991), low copy centromeric vectors that contain autonomously replicating sequences (ARS) such as YRp7 (M. Fitzgerald-Hayes, L. Clarke, and J. Carbon, Cell 29:235,1982) and well as integration vectors that permit the gene of interest to be introduced into specified locus within the host genome and propagated in a stable manner (R.J. Rothstein, Methods in Enzymology 101:202, 1991). Ectopic expression of MMR genes in yeast can be attenuated or completely eliminated at will by a variety of methods, including but not limited to removal from the medium of the specific chemical inducer (e.g deplete galactose that drives expression of the GAL10 promoter in *Saccharomyces cerevisiae* or methanol that drives expression of the AOX1 promoter in *Pichia pastoris*), extra-chromosomally replicating plasmids can be "cured" of expression plasmid by growth of cells under non-selective conditions (e.g. YEp13 harboring cells can be propagated in the presence of leucine,) and cells that have genes inserted into the genome can be grown with chemicals that force the inserted locus to "loop-out" (*e.g.*, integrants that have URA3 can be selected for loss of the inserted gene by growth of integrants on 5-fluoro-orotic acid (J.D. Boeke, F. LaCroute and G.R. Fink. Mol. Gen. Genet. 197:345-346,1984). Whether by withdrawal of inducer or treatment of

yeast cells with chemicals, removal of MMR expression results in the re-establishment of a genetically stable yeast cell-line. Thereafter, the lack of mutant MMR allows the endogenous, wild type MMR activity in the host cell to function normally to repair DNA. The newly generated mutant yeast strains that exhibit novel, selected output traits are suitable for a wide range of commercial processes or for gene/protein discovery to identify new biomolecules that are involved in generating a particular output trait. While it has been documented that MMR deficiency can lead to as much as a 1000-fold increase in the endogenous DNA mutation rate of a host, there is no assurance that MMR deficiency alone will be sufficient to alter every gene within the DNA of the host bacterium to create altered biochemicals with new activity(s). Therefore, the use of chemical mutagens and their respective analogues such as ethidium bromide, EMS, MNNG, MNU, Tamoxifen, 8-Hydroxyguanine, as well as others such as those taught in: Khromov-Borisov, N.N., et.al. (Mutat. Res. 430:55-74, 1999); Ohe, T., et.al. (Mutat. Res. 429:189-199, 1999); Hour, T.C. et.al. (Food Chem. Toxicol. 37:569-579, 1999); Hrelia, P., et.al. (Chem. Biol. Interact. 118:99-111, 1999); Garganta, F., et.al. (Environ. Mol. Mutagen. 33:75-85, 1999); Ukawa-Ishikawa S., et.al. (Mutat. Res. 412:99-107, 1998); www.ehs.utah.edu/ohh/mutagens, etc. can be used to further enhance the spectrum of mutations and increase the likelihood of obtaining alterations in one or more genes that can in turn generate host yeast with a desired new output trait(s). Mismatch repair deficiency leads to hosts with an increased resistance to toxicity by chemicals with DNA damaging activity. This feature allows for the creation of additional genetically diverse hosts when mismatch defective yeast are exposed to such agents, which would be otherwise impossible due to the toxic effects of such chemical mutagens [Colella, G., et.al. (Br. J. Cancer 80:338-343, 1999); Moreland, N.J., et.al. (Cancer Res. 59:2102-2106, 1999); Humbert, O., et.al. (Carcinogenesis 20:205-214, 1999); Glaab, W.E., et.al. (Mutat. Res. 398:197-207, 1998)].

Moreover, mismatch repair is responsible for repairing chemically-induced DNA adducts, therefore blocking this process could theoretically increase the number, types, mutation rate and genomic alterations of a yeastl [Rasmussen, L.J. et.al. (Carcinogenesis 17:2085-2088, 1996); Sledziewska-Gojska, E., et.al. (Mutat. Res. 383:31-37, 1997); and Janion, C. et.al. (Mutat. Res. 210:15-22, 1989)]. In addition to the chemicals listed above, other types of DNA mutagens include ionizing radiation and UV-irradiation, which is known to cause DNA mutagenesis in yeast, can also be used to potentially enhance this process (Lee CC, Lin HK, Lin JK. 1994. A reverse mutagenicity assay for alkylating agents based on a point mutation in the beta-lactamase gene at the active site serine codon. Mutagenesis 9:401-405; Vidal A, Abril N, Pueyo C. 1995. DNA repair by Ogt alkyltransferase influences EMS mutational specificity. Carcinogenesis 16:817-821). These agents, which are extremely toxic to host cells and therefore result in a decrease in the actual pool size of altered yeast cells are more tolerated in MMR defective hosts and in turn permit an enriched spectrum and degree of genomic mutagenesis.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples that will be provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLES

Example 1: Generation of inducible MMR dominant negative allele vectors and yeast cells harboring the expression vectors

Yeast expression constructs were prepared to determine if the human PMS2 related gene (hPMSR2) (Nicolaides et al. Genomics 30(2):195-206) and the human PMS134 gene (Nicolaides NC, Littman SJ, Modrich P, Kinzler KW, Vogelstein B 1998. A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype. Mol Cell Biol 18:1635-1641) are capable of inactivating the yeast MMR activity and thereby increase the overall frequency of genomic hypermutation, a consequence of which is the generation of variant sib cells with novel output traits following host selection. For these studies, a plasmid encoding the hPMS134 cDNA was altered by polymerase chain reaction (PCR). The 5' oligonucleotide has the following structure: 5'-ACG CAT ATG GAG CGA GCT GAG AGC TCG AGT-3' that includes the NdeI restriction site CAT ATG. The 3'-oligonucleotide has the following structure: 5'-GAA TTC TTA TCA CGT AGA ATC GAG ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC AGT TCC AAC CTT CGC CGA TGC-3' that includes an EcoRI site GAA TTC and the 14 amino acid epitope for the V5 antibody. The oligonucleotides were used for PCR under standard conditions that included 25 cycles of PCR (95^{0}C for 1 minute, 55^{0}C for 1 minute, 72^{0}C for 1.5 minutes for 25 cycles followed by 3 minutes at 72^{0}C). The PCR fragment was purified by gel electrophoresis and cloned into pTA2.1 (Invitrogen) by standard cloning methods (Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, 2001), creating the plasmid pTA2.1-hPMS134. pTA2.1-hPMS134 was digested with the restriction enzyme EcoRI to release the insert which was cloned into EcoRI

restriction site of pPIC3.5K (Invitrogen). The following strategy, similar to that described above to clone human PMS134, was used to construct an expression vector for the human related gene PMSR2. First, the hPMSR2 fragment was amplified by PCR to introduce two restriction sites, an NdeI restriction site at the 5'- end and an Eco RI site at the 3'-end of the fragment. The 5'-oligonucleotide that was used for PCR has the following structure: 5'-ACG CAT ATG TGT CCT TGG CGG CCT AGA-3' that includes the NdeI restriction site CAT ATG. The 3'-oligonucleotide used for PCR has the following structure: 5'-GAA TTC TTA TTA CGT AGA ATC GAG ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC CAT GTG TGA TGT TTC AGA GCT-3' that includes an EcoRI site GAA TTC and the V5 epitope to allow for antibody detection. The plasmid that contained human PMSR3 in pBluescript SK (Nicolaides et al. Genomics 30 (2):195-206,1995) was used as the PCR target with the hPMS2-specific oligonucleotides above. Following 25 cycles of PCR (95^{0}C for 1 minute, 55^{0}C for 1 minute, 72^{0}C for 1.5 minutes for 25 cycles followed by 3 minutes at 72^{0}C). The PCR fragment was purified by gel electrophoresis and cloned into pTA2.1 (Invitrogen) by standard cloning methods (Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, 2001), creating the plasmid pTA2.1-hR2. pTA2.1-hR2 was next digested with the restriction enzyme EcoRI to release the insert (there are two EcoRI restriction sites in the multiple cloning site of pTA2.1 that flank the insert) and the inserted into the yeast expression vector pPIC3.5K (Invitrogen).

Pichia pastoris yeast cells were transformed with pPIC3.5K vector, pPIC3.5K-pms134, and pPIC3.5K-hR2 as follows. First, 5ml of YPD (1% yeast extract, 2% bacto-peptone, 1% dextrose) medium was inoculated with a single colony from a YPD plate (same as YPD liquid but add 2% difco-agar to plate) and incubated with shaking overnight at 30^{0}C. The overnight culture was used to inoculate 500ml of YPD medium (200ul of overnight culture) and the culture incubated at 30^{0}C until the optical density at 600nm

reached 1.3 to 1.5. The cells were then spun down (4000 x g for 10 minutes), and then washed 2 times in sterile water (one volume each time), then the cells suspended in 20ml of 1M sorbitol. The sorbitol/cell suspension was spun down (4,000xg for 10 minutes) and suspended in 1ml of 1M sorbitol. 80ul of the cell suspension was mixed with 5 to 10ug of linearized plasmid DNA and placed in a 0.2cm cuvette, pulsed length 5 to 10milliseconds at field strength of 7,500V/cm. Next, the cells are diluted in 1ml of 1M sorbitol and transferred to a 15ml tube and incubated at 30^{0}C for 1 to 2 hours without shaking. Next, the cells are spun out (4,000 x G for 10 minutes) and suspended in 100ul of sterile water, and 50ul/plate spread onto the appropriate selective medium plate. The plates are incubated for 2 to 3 days at 30^{0}C and colonies patched out onto YPD plates for further testing.

Example 2: Generation of hypermutable yeast with inducible dominant negative alleles of mismatch repair genes

Yeast clones expressing human PMS2 homologue PMS-R2 or empty vector were grown in BMG (100mM potassium phosphate, pH 6.0, 1.34% YNB (yeast nitrogen base), 4 x 10^{-5} % biotin, 1% glycerol) liquid culture for 24 hr at 30°C. The next day, cultures were diluted 1:100 in MM medium (1.34% YNB, 4 x 10^{-5}% biotin, 0.5% methanol) and incubated at 30^{0}C with shaking. Cells were removed for mutant selection at 24 and 48 hours post methanol induction as described below (see **EXAMPLE 3**).

EXAMPLE 3: Dominant negative MMR genes can produce new genetic variants and commercially viable output traits in yeast.

The ability to express MMR genes in yeast, as presented in example 2, demonstrate the ability to generate genetic alterations and new phenotypes in yeast expressing dominant negative MMR genes. In this **example** we teach the utility of this method to create eukaryotic strains with commercially relevant output traits.

GENERATION OF URACIL DEPENDENT YEAST STRAIN

One example of utility is the generation of a yeast strain that is mutant for a particular metabolic product, such as an amino acid or nucleotide. Engineering such a yeast strain will allow for recombinant manipulation of the yeast strain for the introduction of genes for scalable process of recombinant manufacturing. In order to demonstrate that MMR can be manipulated in yeast to generate mutants that lack the abilty to produce specific molecular building blocks, the following experiment was performed. Yeast cells that express a methanol inducible human PMS2 homologue, hPMS2-R2 (as described in example 1 above), were grown in BMY medium overnight then diluted 1:100 and transferred to MM medium, which results in activation of the AOX promoter and production of the hPMS2-R2 MMR gene that is resident within the yeast cell. Control cells were treated the same manner; these cells contain the pPIC3.5 vector in yeast and lack an insert. Cells were induced for 24 and 48 hours and then selected for uracil requiring mutations as follows. The cells were plated to 5-FOA medium (Boeke, J.D., LaCroute, F., and Fink, G.R. Mol. Gen. Genet. 197:345-345,1984). The plates are made as follows: (2X concentrate (filter sterilize): yeast nitrogen base 7 grams; 5-fluoro-orotic acid 1 gram; uracil 50 milligrams; glucose 20 grams; water to 500 ml; Add to 500 ml 4% agar (autoclaved) and pour plates. Cells are plated on 5-FOA plates (0, 24 and 48 hour time points) and incubated at 30^{0}C for between 3 and 5 days. Data from a typical experiment is shown in Table 1. No uracil requiring clones were observed in the un-induced or induced culture in yeast cells that harbor the "empty" vector whereas those cells that harbor the MMR gene hPMS2-R2 have clones that are capable of growth on the selection medium. Note that the un-induced culture of hPMS2-R2 does not have any colonies that are resistant to 5-FOA,

demonstrating that the gene must be induced for the novel phenotype to be generated. It has been demonstrated that the mutagens (such as ethyl methyl sulfonate result in a low number of ura⁻ mutants and that the spontaneous mutation rate for generating this class of mutants is low (Boeke, J.D., LaCroute, F. and Fink, G.R. Mol. Gen. Genet. 197:345-346,1984).

Table 1: Generation of uracil requiring mutant *Pichia pastoris* yeast cells. # Represents at 24 hour methanol induction and @ a 48 hour induction. For comparison a wild type yeast cell treated/un-treated is shown (Galli, A. and Schiestl, R.H. Mutat. Res. 429(1):13-26,1999).

Strain	Seeded	ura^-	URA^+	Frequency (ura^- cells)
Wt	100,000	0	~100,000	0
Empty	100,000	0	~100,000	0
$pMOR^{ye-1\#}$	100,000	14	~100,000	1/7,142
$pMOR^{ye2@}$	100,000	123	~100,000	1/813
Wt	100,000	1-0.1	100,000	$1/10^{5-6*}$
Mutagen	100,000	10	100,000	1/10,000

GENERATION OF HEAT-RESISTANT PRODUCER STRAINS

One example of commercial utility is the generation of heat-resistant recombinant protein producer strains. In the scalable process of recombinant manufacturing, large-scale fermentation of both prokaryotes and eukaryotes results in the generation of excessive heat within the culture. This heat must be dissipated by physical means such as using cooling jackets that surround the culture while it is actively growing and producing product. Production of a yeast strain that can resist high temperature growth effectively would be advantageous for large-scale recombinant manufacturing processes. To this end, the yeast strain as described in

EXAMPLE 2 can be grown in the presence of methanol to induce the dominant negative MMR gene and the cells grown for various times (e.g. 12, 24, 36 and 48 hours) then put on plates and incubated at elevated temperatures to select for mutants that resist high temperature growth (e.g. 37^{0}C or 42^{0}C). These strains would be useful for fermentation development and scale-up of processes and should result in a decrease in manufacturing costs due to the need to cool the fermentation less often.

GENERATION OF HIGH RECOMBINANT PROTEIN PRODUCER STRAINS AND STRAINS WITH LESS ENDOGENOUS PROTEASE ACTIVITY

Yeast is a valuable recombinant-manufacturing organism since it is a single celled organism that is inexpensive to grow and easily lends itself to fermentation at scale. Further more, many eukaryotic proteins that are incapable of folding effectively when expressed in *Escherichia coli* systems fold with the proper conformation in yeast and are structurally identical to their mammalian counterparts. There are several inherent limitations of many proteins that are expressed in yeast including over and/or inappropriate glycosylation of the recombinant protein, proteolysis by endogenous yeast enzymes and insufficient secretion of recombinant protein from the inside of the yeast cell to the medium (which facilitates purification). To generate yeast cells that with this ability to over-secrete proteins, or with less endogenous protease activity and or less hyper-glycosylation activity yeast cells as described in example 1 can be grown with methanol for 12, 24, 36 and 48 hours and yeast cells selected for the ability to over-secrete the protein or interest, under-glycosylate it or a cell with attenuated of no protease activity. Such a strain will be useful for recombinant manufacturing or other commercial purposes and can be combined with the heat resistant strain outlined above. For example, a mutant yeast cell that is resistant to high temperature growth and can secrete large amounts of protein into the medium would result.

Similar results were observed with other dominant negative mutants such as the PMSR2, PMSR3, and the human MLH1 proteins.

EXAMPLE 4: Mutations generated in the host genome of yeast by defective MMR are genetically stable

As described in example 3 manipulation of the MMR pathway in yeast results in alterations within the host genome and the ability to select for a novel output traits, for example the ability of a yeast cell to require a specific nutrient. It is important that the mutations introduced by the MMR pathway is genetically stable and passed to daughter cells reproducibly once the wild type MMR pathway is re-established. To determine the genetic stability of mutations introduced into the yeast genome the following experiment was performed. Five independent colonies from pPIC3.5K-hPMS2-R2 that are ura$^-$, five wild type control cells (URA$^+$) and five pPIC3.5K transformed cells ("empty vector") were grown overnight from an isolated colony in 5 ml of YPD (1% yeast extract, 2% bacto-peptone and 1% dextrose) at 30^{0}C with shaking. The YPD medium contains all the nutrients necessary for yeast to grow, including uracil. Next, 1μL of the overnight culture, which was at an optical density (OD) as measured at 600nM of >3.0, was diluted to an OD_{600} of 0.01 in YPD and the culture incubated with shaking at 30^{0}C for an additional 24 hours. This process was repeated 3 more times for a total of 5 overnight incubations. This is the equivalent of greater than 100 generations of doublings (from the initial colony on the plate to the end of the last overnight incubation. Cells (five independent colonies that are ura$^-$ and five that were wild type were then plated onto YPD plates at a cell density of 300 to 1,000 cells/plate and incubated for two days at 30^{0}C. The cells from these plates were replica plated to the following plates and scored for growth following three days incubation at 30^{0}C; <u>S</u>ynthetic <u>C</u>omplete (SC) SC-ura (1.34% yeast nitrogen base and ammonium sulfate; 4 x 10^{-5} % biotin; supplemented with all amino acids, NO supplemental uracil; 2% dextrose and 2% agar); SC +URA (same as SC-ura but supplement plate with 50 mg uracil/liter medium), and YPD plates. They were replica plated in the following order- SC –ura, SC complete, YPD. If the novel output trait

that is resident within the yeast genome that was generated by expression of the mutant MMR (in this example the human homologue of PMS2, hPMS2-R2) is unstable, the uracil dependent cells should "revert" back a uracil independent phenotype. If the phenotype is stable, growth of the mutant cells under non-selective conditions should result in yeast cells that maintain their viability dependence on exogenous supplementation with uracil. As can be seen in the data presented in Table 2, the uracil dependent phenotype is stable when the yeast cells are grown under non-selective conditions, demonstrating that the MMR-generated phenotype derived from mutation in one of the uracil biosynthetic pathway genes is stable genetically.

Strain	Seeded	-ura	+URA	YPD
Wt	650	650	650	650
Empty	560	560	560	560
pMOR$^{ye\text{-}1\#}$	730	0	730	730

These data demonstrate the utility of employing an inducible expression system and a dominant negative MMR gene in a eukaryotic system to generate genetically altered strains. The strain developed in this example, a yeast strain that now requires addition of uracil for growth, is potentially useful as a strain for recombinant manufacturing; by constructing an expression vector that harbors the wild type URA3 gene on either an integration plasmid or an extra-chromosomal vector it is now possible to transform and create novel cells expressing the a protein of interest. It is also possible to modify other resident genes in yeast cells and select for mutations in genes that that give other useful phenotypes, such as the ability to carry out a novel bio-transformation. Furthermore, it is possible to express a gene extra-chromosomally in a yeast cell that has altered MMR activity as described above and select for mutations in the extra-chromosomal gene. Therefore, in a similar manner to that described above the mutant yeast cell can be put under specific selective pressure and a novel protein with commercially important biochemical attributes selected. These examples are meant only as illustrations and are not meant to limit the scope of the present invention.

Finally, as described above once a mutation has been introduced into the gene of interest the MMR activity is attenuated of completely abolished. The result is a yeast cell that harbors a stable mutation in the target gene(s) of interest.

EXAMPLE 5: Enhanced Generation of MMR-Defective Yeast and Chemical Mutagens for the Generation of New Output Traits

It has been previously documented that MMR deficiency yields to increased mutation frequency and increased resistance to toxic effects of chemical mutagens (CM) and their respective analogues such as but not limited to those as: ethidium bromide, EMS, MNNG, MNU, Tamoxifen, 8-Hydroxyguanine, as well as others listed but not limited to in publications by: Khromov-Borisov, N.N., et.al. Mutat. Res. 430:55-74, 1999; Ohe, T., et.al. (Mutat. Res. 429:189-199, 1999; Hour, T.C. et.al. Food Chem. Toxicol. 37:569-579, 1999; Hrelia, P., et.al. Chem. Biol. Interact. 118:99-111, 1999; Garganta, F., et.al. Environ. Mol. Mutagen. 33:75-85, 1999; Ukawa-Ishikawa S., et.al. Mutat. Res. 412:99-107, 1998; www.ehs.utah.edu/ohh/mutagens; Marcelino LA, Andre PC, Khrapko K, Coller HA, Griffith J, Thilly WG. Chemically induced mutations in mitochondrial DNA of human cells: mutational spectrum of N-methyl-N'-nitro-N-nitrosoguanidine. *Cancer Res* 1998 Jul 1;58(13):2857-62; Koi M, Umar A, Chauhan DP, Cherian SP, Carethers JM, Kunkel TA, Boland CR. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Can res 1994 54:4308-4312,1994. Mismatch repair provokes chromosome aberrations in hamster cells treated with methylating agents or 6-thioguanine, but not with ethylating agents. To demonstrate the ability of CMs to increase the mutation frequency in MMR defective yeast cells, we would predict that exposure of yeast cells to CMs in the presence or absence of methanol (which induces the expression of the resident human homologue to PMS2, hPMS2-R2) will result in an augmentation of mutations within the yeast cell.

Yeast cells that express hPMS2-R2 (induced or un-induced) and empty vector control cells are grown as described in examples 2 and 3) and for 24 hours and diluted into MM

medium as described above. Next, the cells in MM are incubated either with or without increasing amounts of ethyl methane sulfonate (EMS) from 0, 1, 10, 50, 100, and 200 μM. 10 μL aliquots of culture (diluted in 300 μl MM) and incubated for 30 minutes, 60 minutes, and 120 minutes followed by plating cells onto 5-FOA plates as described in example 3 above. Mutants are selected and scored as above. We would predict that there will be an increase in the frequency of ura⁻ mutants in the PMS2-R2 cultures that are induced with methanol as compared to the uninduced parental or wild type strain. In a further extension of this example, human PMS2-R2 harboring cells will be induced for 24 and 48 hours then mutagenized with EMS. This will allow the MMR gene to be fully active and expressed at high levels, thereby resulting in an increase in the number of ura⁻ mutants obtained. We would predict that there will be no change in the number of ura⁻ mutants obtained in the un-induced parental control or the wild type "empty vector" cells.

This example demonstrates the use of employing a regulated dominant negative MMR system plus chemical mutagens to produce enhanced numbers of genetically altered yeast strains that can be selected for new output traits. This method is useful for generating such organisms for commercial applications such as but not limited to recombinant manufacturing, biotransformation, and altered biochemicals with enhanced activities. It is also useful to obtain alterations of protein activity from ectopically expressed proteins harbored on extra-chromosomal expression vectors similar to those described in example 4 above.

EXAMPLE 6: Alternative Methods to Inhibition of Yeast MMR Activity

The inhibition of MMR activity in a host organism can be achieved by introducing a dominant negative allele as shown in the examples above. This application also teaches us the use of using regulated systems to control MMR in yeast to generate genetic diversity and output traits for commercial applications. Additional methods to regulate the suppression of MMR activity of a host are by using genetic recombination to knock out alleles of a MMR gene within the cell of interest. This can be accomplished by use of homologous recombination that disrupts the endogenous MMR gene; 2) blocking MMR protein

dimerization with other subunits (which is required for activity) by the introduction of polypeptides or antibodies into the host via transfection methods routinely used by those skilled in the art (e.g. electroporation); or 3) decreasing the expression of a MMR gene using anti-sense oligonucleotides.

MMR gene knockouts. We intend to generate disrupted targeting vectors of a particular MMR gene and introduce it into the genome of yeast using methods standard in the art. Yeast exhibiting hypermutability will be useful to produce genetically diverse offspring for commercial applications. Yeast will be confirmed to have lost the expression of the MMR gene using standard northern and biochemical techniques (as described in reference 31). MMR gene loci can be knocked out, strains selected for new output traits and MMR restored by introducing a wild type MMR gene to complement the KO locus. Other strategies include using KO vectors that can target a MMR gene locus, select for host output traits and then have the KO vector "spliced" from the genome after strain generation.

Blocking peptides. MMR subunits (MutS and MutL proteins) interact to form active MMR complexes. Peptides are able to specifically inhibit the binding of two proteins by competitive inhibition. Introduction into cells of peptides or antibodies to conserved domains of a particular MMR gene to disrupt activity is straightforward to those skilled in the art. Yeast will be verified for loss of expression of the MMR activity by standard northern and/or biochemical techniques (as described in Nicolaides NC, Littman SJ, Modrich P, Kinzler KW, Vogelstein B 1998. A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype. Mol Cell Biol 18:1635-1641). Yeast exhibiting hypermutability will be useful to produce genetically diverse sibs for commercial applications.

Discussion

The results described above will lead to several conclusions. First, expression of dominant negative MMR proteins results in an increase in microsatellite instability and hypermutability in yeast. The hypermutability of the yeast cell is due to the inhibition of the resident, endogenous MMR biochemical activity in these hosts. This method provides a claim

for use of MMR genes and their encoded products for the creation of hypermutable yeast to produce new output traits for commercial applications.

EXAMPLES OF MMR GENES AND ENCODED POLYPEPTIDES

Yeast MLH1 cDNA (accession number U07187)

```
   1 aaataggaat gtgatacctt ctattgcatg caaagatagt gtaggaggcg ctgctattgc
  61 caaagacttt tgagaccgct tgctgtttca ttatagttga ggagttctcg aagacgagaa
 121 attagcagtt ttcggtgttt agtaatcgcg ctagcatgct aggacaattt aactgcaaaa
 181 ttttgatacg atagtgatag taaatggaag gtaaaaataa catagaccta tcaataagca
 241 atgtctctca gaataaaagc acttgatgca tcagtggtta acaaaattgc tgcaggtgag
 301 atcataatat cccccgtaaa tgctctcaaa gaaatgatgg agaattccat cgatgcgaat
 361 gctacaatga ttgatattct agtcaaggaa ggaggaatta aggtacttca aataacagat
 421 aacggatctg gaattaataa agcagacctg ccaatcttat gtgagcgatt cacgacgtcc
 481 aaattacaaa aattcgaaga tttgagtcag attcaaacgt atggattccg aggagaagct
 541 ttagccagta tctcacatgt ggcaagagtc acagtaacga caaaagttaa agaagacaga
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 781 gccattcatt ccaaggacat tggcttttct tgtaaaaagt tcggagactc taattattct
 841 ttatcagtta aaccttcata tacagtccag gataggatta ggactgtgtt caataaatct
 901 gtggcttcga atttaattac ttttcatatc agcaaagtag aagatttaaa cctggaaagc
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3181 aacaataaga ctaatgatta ccttagaata atgtataa

Yeast MLH1 protein (accession number U07187)

MSLRIKALDASVVNKIAAGEIIISPVNALKEMMENSIDANATMI
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Mouse PMS2 protein

MEQTEGVSTE CAKAIKPIDG KSVHQICSGQ VILSLSTAVK ELIENSVDAG ATTIDLRLKD 60
YGVDLIEVSD NGCGVEEENF EGLALKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
TISTCHGSAS VGTRLVFDHN GKITQKTPYP RPKGTTVSVQ HLFYTLPVRY KEFQRNIKKE 180
YSKMVQVLQA YCIISAGVRV SCTNQLGQGK RHAVVCTSGT SGMKENIGSV FGQKQLQSLI 240
PFVQLPPSDA VCEEYGLSTS GRHKTFSTFR ASFHSARTAP GGVQQTGSFS SSIRGPVTQQ 300
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LREAFSLHPT KEIKSRGPET AELTRSFPSE KRGVLSSYPS DVISYRGLRG SQDKLVSPTD 480
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EVDVAIKMNK RIVLLEFSLS SLAKRMKQLQ HLKAQNKHEL SYRKFRAKIC PGENQAAEDE 660
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PHGRPTMRHV ANLDVISQN 859

Mouse PMS2 cDNA

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human PMS2 protein

MKQLPAATVR LLSSSQIITS VVSVVKELIE NSLDAGATSV DVKLENYGFD KIEVRDNGEG 60
IKAVDAPVMA MKYYTSKINS HEDLENLTTY GFRGEALGSI CCIAEVLITT RTAADNFSTQ 120
YVLDGSGHIL SQKPSHLGQG TTVTALRLFK NLPVRKQFYS TAKKCKDEIK KIQDLLMSFG 180
ILKPDLRIVF VHNKAVIWQK SRVSDHKMAL MSVLGTAVMN NMESFQYHSE ESQIYLSGFL 240
PKCDADHSFT SLSTPERSFI FINSRPVHQK DILKLIRHHY NLKCLKESTR LYPVFFLKID 300
VPTADVDVNL TPDKSQVLLQ NKESVLIALE NLMTTCYGPL PSTNSYENNK TDVSAADIVL 360
SKTAETDVLF NKVESSGKNY SNVDTSVIPF QNDMHNDESG KNTDDCLNHQ ISIGDFGYGH 420
CSSEISNIDK NTKNAFQDIS MSNVSWENSQ TEYSKTCFIS SVKHTQSENG NKDHIDESGE 480
NEEEAGLENS SEISADEWSR GNILKNSVGE NIEPVKILVP EKSLPCKVSN NNYPIPEQMN 540
LNEDSCNKKS NVIDNKSGKV TAYDLLSNRV IKKPMSASAL FVQDHRPQFL IENPKTSLED 600
ATLQIEELWK TLSEEEKLKY EEKATKDLER YNSQMKRAIE QESQMSLKDG RKKIKPTSAW 660
NLAQKHKLKT SLSNQPKLDE LLQSQIEKRR SQNIKMVQIP FSMKNLKINF KKQNKVDLEE 720
KDEPCLIHNL RFPDAWLMTS KTEVMLLNPY RVEEALLFKR LLENHKLPAE PLEKPIMLTE 780
SLFNGSHYLD VLYKMTADDQ RYSGSTYLSD PRLTANGFKI KLIPGVSITE NYLEIEGMAN 840
CLPFYGVADL KEILNAILNR NAKEVYECRP RKVISYLEGE AVRLSRQLPM YLSKEDIQDI 900
IYRMKHQFGN EIKECVHGRP FFHHLTYLPE TT 932

Human PMS2 cDNA

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human PMS1 protein

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Human PMS1 cDNA

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human MSH2 protein
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Human MSH2 cDNA

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human MLH1 protein

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DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL 660
ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV 720

YKALRSHILP PKHFTEDGNI LQLANLPDLY KVFERC 756

Human MLH1 cDNA

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gagtcgaccc tctcaggcca gcagagtgaa gtgcctggct ccattccaaa ctcctggaag 2160
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cgatacaaag tgttgtatca aagtgtgata tacaaagtgt accaacataa gtgttggtag 2400
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aataaataga tgtgtcttaa cata 2484

hPMS2-134 protein
MKQLPAATVR LLSSSQIITS VVSVVKELIE NSLDAGATSV DVKLENYGFD KIEVRDNGEG 60
IKAVDAPVMA MKYYTSKINS HEDLENLTTY GFRGEALGSI CCIAEVLITT RTAADNFSTQ 120
YVLDGSGHIL SQK 133

hPMS2-134 cDNA
cgaggcggat cgggtgttgc atccatggag cgagctgaga gctcgagtac agaacctgct 60
aaggccatca aacctattga tcggaagtca gtccatcaga tttgctctgg gcaggtggta 120
ctgagtctaa gcactgcggt aaaggagtta gtagaaaaca gtctggatgc tggtgccact 180
aatattgatc taaagcttaa ggactatgga gtggatctta ttgaagtttc agacaatgga 240
tgtggggtag aagaagaaaa cttcgaaggc ttaactctga aacatcacac atctaagatt 300
caagagtttg ccgacctaac tcaggttgaa acttttggct ttcgggggga agctctgagc 360
tcactttgtg cactgagcga tgtcaccatt tctacctgcc acgcatcggc gaaggttgga 420
acttga 426

hMSH6 (human cDNA) ACCESSION U28946

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ASTLYVPEDFLNSCTPGMRKWWQIKSQNFDLVICYKVGKFYELYHMDALIGVSELGLV
FMKGNWAHSGFPEIAFGRYSDSLVQKGYKVARVEQTETPEMMEARCRKMAHISKYDRV
VRREICRIITKGTQTYSVLEGDPSENYSKYLLSLKEKEEDSSGHTRAYGVCFVDTSLG
KFFIGQFSDDRHCSRFRTLVAHYPPVQVLFEKGNLSKETKTILKSSLSCSLQEGLIPG
SQFWDASKTLRTLLEEEYFREKLSDGIGVMLPQVLKGMTSESDSIGLTPGEKSELALS
ALGGCVFYLKKCLIDQELLSMANFEEYIPLDSDTVSTTRSGAIFTKAYQRMVLDAVTL
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DFLSALEGFKVMCKIIGIMEEVADGFKSKILKQVISLQTKNPEGRFPDLTVELNRWDT
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GRNRYQLEIPENFTTRNLPEEYELKSTKKGCKRYWTKTIEKKLANLINAEERRDVSLK
DCMRRLFYNFDKNYKDWQSAVECIAVLDVLLCLANYSRGGDGPMCRPVILLPEDTPPF

LELKGSRHPCITKTFFGDDFIPNDILIGCEEEEQENGKAYCVLVTGPNMGGKSTLMRQ
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TAHSLVLVDELGRGTATFDGTAIANAVVKELAETIKCRTLFSTHYHSLVEDYSQNVAV
RLGHMACMVENECEDPSQETITFLYKFIKGACPKSYGFNAARLANLPEEVIQKGHRKA
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hPMSR2 (human cDNA) ACCESSION U38964

```
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  61 ttggggagcc agtacatgca ggtgggctcc acacggagag gggcgcagac ccggtgacag
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 361 gcagtccggt gtgctctgat tggtccaggc tctttgacgt cacggactcg acctttgaca
 421 gagccactag gcgaaaagga gagacgggaa gtatttttc cgccccgccc ggaaagggtg
 481 gagcacaacg tcgaaagcag ccgttgggag cccaggaggc ggggcgcctg tgggagccgt
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 721 aacagtctgg atgctggtgc cactaatgtt gatctaaagc ttaaggacta tggagtggat
 781 ctcattgaag tttcaggcaa tggatgtggg gtagaagaag aaaacttcga aggctttact
 841 ctgaaacatc acacatgtaa gattcaagag tttgccgacc taactcaggt ggaaactttt
 901 ggctttcggg gggaagctct gagctcactt tgtgcactga gtgatgtcac catttctacc
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1021 cagaaaaccc cctacccccg ccccagaggg atgacagtca gcgtgaagca gttattttct
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1381 aatccaaaaa aaaaaaaaaa aaaaaaa
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hPMSR2 (human protein) ACCESSION U38964

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EVFFPPRPERVEHNVESSRWEPRRRGACGSRGGNFPSPRGGSGVASLERAENSSTEPA

KAIKPIDRKSVHQICSGPVVPSLRPNAVKELVENSLDAGATNVDLKLKDYGVDLIEVS

GNGCGVEEENFEGFTLKHHTCKIQEFADLTQVETFGFRGEALSSLCALSDVTISTCRV

SAKVGTRLVFDHYGKIIQKTPYPRPRGMTVSVKQLFSTLPVHHKEFQRNIKKKRACFP

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HPMSR3 (human cDNA) ACCESSION U38979

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 121 gggagctgca gaccagtctt ccgtggcagg ctgagcgctc cagtcttcag tagggaattg
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 241 agcagtgaac tcaggagcgg gagcagtcca ttcaccctga aattcctcct tggtcactgc
 301 cttctcagca gcagcctgct cttctttttc aatctcttca ggatctctgt agaagtacag
 361 atcaggcatg acctcccatg ggtgttcacg ggaaatggtg ccacgcatgc gcagaacttc
 421 ccgagccagc atccaccaca ttaaacccac tgagtgagct cccttgttgt tgcatgggat
 481 ggcaatgtcc acatagcgca gaggagaatc tgtgttacac agcgcaatgg taggtaggtt
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 661 gcccccagaa ggacacccgc ctggatttgc cccgtagccc ggcccgggcc cctcgggagc
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 961 accctgatga gaagatagca tacggggatg tgatgttgga gaactacagc catctagttt
1021 ctgtggggta tgattatcac caagccaaac atcatcatgg agtggaggtg aaggaagtgg
1081 agcagggaga ggagccgtgg ataatggaag gtgaatttcc atgtcaacat agtccagaac
1141 ctgctaaggc catcaaacct attgatcgga agtcagtcca tcagatttgc tctgggccag
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1681 ctgccgtgat tgtcagtttc ctgaggcctc cccagccatg cttcctgtac agcctgcaga
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hPMSR3 (human protein) ACCESSION U38979

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VEQGEEPWIMEGEFPCQHSPEPAKAIKPIDRKSVHQICSGPVVLSLSTAVKELVENSL
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hPMSL9 (human cDNA) ACCESSION NM_005395

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   1 atgtgtcctt ggcggcctag actaggccgt cgctgtatgg tgagccccag ggaggcggat
  61 ctgggccccc agaaggacac ccgcctggat ttgccccgta gcccggcccg ggcccctcgg
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 181 gcgacagcag ccccgccccg gcctctcggg agccgggggg cagaggctgc ggagccccag
 241 gagggtctat cagccacagt ctctgcatgt ttccaagagc aacaggaaat gaacacattg
 301 caggggccag tgtcattcaa agatgtggct gtggatttca cccaggagga gtggcggcaa
 361 ctggaccctg atgagaagat agcatacggg gatgtgatgt tggagaacta cagccatcta
 421 gtttctgtgg ggtatgatta tcaccaagcc aaacatcatc atggagtgga ggtgaaggaa
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 541 gaacctgcta aggccatcaa acctattgat cggaagtcag tccatcagat ttgctctggg
 601 ccagtggtac tgagtctaag cactgcagtg aaggagttag tagaaaacag tctggatgct
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661 ggtgccacta atattgatct aaagcttaag gactatggag tggatctcat tgaagtttca
721 gacaatggat gtggggtaga agaagaaaac tttgaaggct taatctcttt cagctctgaa
781 acatcacaca tgtaa
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hPMSL9 (human protein) ACCESSION NM_005395

MCPWRPRLGRRCMVSPREADLGPQKDTRLDLPRSPARAPREQNS
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WE CLAIM:

1. A method for making a hypermutable yeast, comprising the step of: introducing into a yeast a polynucleotide comprising a dominant negative allele of a mismatch repair gene, whereby the cell becomes hypermutable.

2. The method of claim 1 wherein the mismatch repair gene is a MutH homolog.

3. The method of claim 1 wherein the mismatch repair gene is a MutS homolog.

4. The method of claim 1 wherein the mismatch repair gene is a MutL homolog.

5. The method of claim 1 wherein the mismatch repair gene is a MutY homolog.

6. The method of claim 1 wherein the mismatch repair gene is *PMS2*.

7. The method of claim 1 wherein the mismatch repair gene is plant *PMS2*.

8. The method of claim 1 wherein the mismatch repair gene is *MLH1*.

9. The method of claim 1 wherein the mismatch repair gene is *MLH3*.

10. The method of claim 1 wherein the mismatch repair gene is *MSH2*.

11. The method of claim 1 wherein the mismatch repair gene is a *PMSR* or *PMSL* homolog.

12. The method of claim 4 wherein the allele comprises a truncation mutation.

13. The method of claim 6 where the allele comprises a truncation mutation.

14. The method of claim 7 where the allele comprises a truncation mutation.

15. The method of claim 3 where the allele comprises a truncation mutation.

16. The method of claim 3 wherein the allele comprises a truncation mutation at codon 134.

17. The method of claim 4 wherein the allele comprises a truncation mutation at codon 134.

18. The method of claim 6 wherein the allele comprises a truncation mutation at codon 134.

19. The method of claim 1 wherein the polynucleotide is introduced into a yeast by mating.

20. The method of claim 6 wherein the mismatch repair gene is mammalian *PMS2*.

21. The method of claim 14 wherein the mismatch repair gene is plant *PMS2*.

22. The method of claim 12 wherein the mismatch repair gene is *MLH1*.

23. The method of claim 12 wherein the mismatch repair gene is *MLH3*.

24. The method of claim 15 wherein the mismatch repair gene is *MSH2*.

25. The method of claim 15 wherein the mismatch repair gene is *MSH3*.

26. The method of claim 15 wherein the mismatch repair gene is *MSH6*.

27. The method of claim 12 wherein the mismatch repair gene is a plant MutL homolog.

28. A homogeneous composition of cultured, hypermutable, yeast which comprise a dominant negative allele of a mismatch repair gene.

29. The isolated hypermutable yeast of claim 28 wherein the mismatch repair gene is a mutL gene or homolog.

30. The isolated hypermutable yeast of claim 28 wherein the mismatch repair gene is a *PMS2* gene or homolog.

31. The isolated hypermutable yeast of claim 28 wherein the mismatch repair gene is a *MLH1* or homolog.

32. The isolated hypermutable yeast of claim 28 wherein the mismatch repair gene is a *PMSR homolog*.

33. The isolated hypermutable yeast of claim 28 wherein the mismatch repair gene is *mutS* or a homolog.

34. The isolated hypermutable yeast of claim 28 wherein the mismatch repair gene is eukaryotic.

35. The isolated hypermutable yeast of claim 28 wherein the mismatch repair gene is procaryotic.

36. The isolated hypermutable yeast of claim 30 wherein the cells express a protein consisting of the first 133 amino acids of PMS2.

37. The isolated hypermutable yeast of claim 30 comprising a protein which consists of the first 133 amino acids of PMS2.

38. The isolated hypermutable yeast of claim 33 comprising a mammalian MutS protein.

39. The isolated hypermutable yeast of claim 31 comprising a protein which consists of a mammalian MutL protein.

40. The isolated hypermutable yeast of claim 28 comprising a eukaryotic MutL protein.

41. The isolated hypermutable yeast of claim 28 comprising a eukaryotic MutS protein.

42. A method for generating a mutation in a gene of interest comprising the steps of:

growing a yeast culture comprising the gene of interest and a dominant negative allele of a mismatch repair gene, wherein the cell is hypermutable; and

testing the cell to determine whether the gene of interest harbors a mutation.

43. The method of claim 42 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

44. The method of claim 42 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

45. The method of claim 42 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

46. The method of claim 42 wherein the step of testing comprises analyzing a phenotype associated with the gene of interest.

47. The method of claim 42 wherein the yeast is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a yeast cell, whereby the yeast cell becomes hypermutable.

48. The method of claim 47 wherein the step of testing comprises analyzing the nucleotide sequence from the gene of interest.

49. The method of claim 47 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

50. The method of claim 47 wherein the step of testing comprises analyzing the phenotype of the gene of interest.

51. A method for generating a mutation in a gene of interest comprising the steps of:

growing a yeast cell comprising the gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene to create a population of mutated, hypermutable yeast cells;

cultivating the population of mutated, hypermutable yeast cells under trait selection conditions;

testing the yeast cells which grow under trait selection conditions to determine whether the gene of interest harbors a mutation.

52. The method of claim 51 wherein the step of testing comprises analyzing a nucleotide sequence of the gene of interest.

53. The method of claim 51 wherein the step of testing comprises analyzing mRNA transcribed from the gene of interest.

54. The method of claim 51 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

55. The method of claim 51 wherein the step of testing comprises analyzing a phenotype associated with the gene of interest.

56. The method of claim 51 further comprising the step of using the yeast cells which harbor a mutation in the gene of interest to produce a recombinant product.

57. The method of claim 51 further comprising the step of using the yeast cells which harbor a mutation in the gene of interest to perform a biotransformation.

58. The method of claim 51 further comprising the step of using the yeast cells which harbor a mutation in the gene of interest to perform bioremediation.

59. The method of claim 51 further comprising the step of using the yeast cells which harbor a mutation in the gene of interest to identify genes encoding viral antigens.

60. The method of claim 51 further comprising the step of using the yeast cells which harbor a mutation in the gene of interest to identify yeast antigens.

61. The method of claim 51 further comprising the step of using the yeast cells which harbor a mutation in the gene of interest to identify pharmaceutical targets.

62. The method of claim 51 wherein themutation in the gene of interest causes antibiotic resistance, and the gene is cloned.

63. The method of claim 51 further comprising the step of using the yeast cells which harbor a mutation in the gene of interest to screen compund libraries.

64. A method for generating enhanced hypermutable yeast comprising the steps of:

exposing a yeast cell to a mutagen, wherein the yeast cell is defective in mismatch repair (MMR) due to the presence of a dominant negative allele of at least one MMR gene, whereby an enhanced rate of mutation of the yeast cell is achieved.

65. The method of claim 64 wherein the mutagen is a DNA alkylating agent.

66. The method of claim 64 wherein the mutagen is a DNA intercalating agent.

67. The method of claim 64 wherein the mutagen is a DNA oxidizing agent.

68. The method of claim 64 wherein the mutagen is ionizing radiation.

69. The method of claim 64 wherein the mutagen is ultraviolet irradiation.

70. The method of claim 64 wherein the dominant negative allele is inducibly regulated.

71. A method for generating mismatch repair (MMR)-proficient yeast with new output traits, comprising the steps of:

growing a yeast cell comprising a gene of interest and a polynucleotide encoding a dominant negative allele of a mismatch repair gene to create a population of mutated, hypermutable yeast cells;

cultivating the population of mutated, hypermutable yeast cells under trait selection conditions;

testing the yeast cells which grow under trait selection conditions to determine whether the gene of interest harbors a mutation;

restoring normal mismatch repair activity to the yeast cells..

72. The method of claim 71 wherein the yeast cell is exposed to a mutagen to increase the rate of mutation prior to the step of cultivating.

73. The method of claim 71 wherein the step of restoring normal mismatch repair activity comprises removing an inducer which regulates transcription of the dominant negative allele from the yeast cells.

74. The method of claim 72 wherein the step of restoring normal mismatch repair activity comprises removing an inducer which regulates transcription of the dominant negative allele from the yeast cells.

75. The method of claim 73 wherein the inducer is methanol.

76. The mthod of claim 73 wherein the inducer is galactose.

77. The method of claim 71 wherein the step of restoring normal mismatch repair activity comprises excising the dominant negative allele by homologous recombination.

78. The method of claim 71 wherein the step of restoring normal mismatch repair activity involves inactivating the dominant negative allele.

79. The method of claim 71 wherein the step of restoring normal mismatch repair activity comprises applying selection conditions to the yeast cells under which cells which have lost the dominant negative allele can grow but cells which harbor the dominant negative allele cannot grow.

80. The method of claim 71 wherein the step of restoring normal mismatch repair activity is performed subsequent to the step of cultivating under trait selection conditions.

81. The method of claim 72 wherein the step of restoring normal mismatch repair activity is performed susequent to the step of exposing to a mutagen and subsequent to the step of cultivating under trait selection conditions

82. The method of claim 72 wherein mutagen is ionizing radiation.

83. The method of claim 72 wherein the mutagen is ultraviolet (UV) irradiation.

84. The method of claim 71 wherein normal mismatch repair activity is restored by complementing with a wild-type mismatch repair allele.

INTERNATIONAL SEARCH REPORT

International Application No
PC1/US 01/05447

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/81 C12N5/10 C12N15/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, MEDLINE, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 240 609 A (THE JOHNS HOPKINS UNIVERSITY, US) 14 October 1999 (1999-10-14) the whole document	1-84
P,X	& US 6 146 894 A (NICHOLAS NICOLAIDES ET AL.) 14 November 2000 (2000-11-14) the whole document ---	1,6,8, 10,11, 13,18, 20,28, 30-32, 34,36, 37,42-71

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2001	10/07/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	NICOLAIDES NICHOLAS C ET AL: "A naturally occurring hPMS2 mutation can confer a dominant negative mutator phenotype" MOLECULAR AND CELLULAR BIOLOGY,AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON,US, vol. 18, no. 3, March 1998 (1998-03), pages 1635-1641, XP002165242 ISSN: 0270-7306 cited in the application abstract page 1636, right-hand column, paragraph 4 -page 1640, right-hand column, paragraph 1	1-84
Y	POLACZEK P ET AL: "Functional genetic tests of DNA mismatch repair protein activity in Saccharomyces cerevisiae" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES,GB,ELSEVIER SCIENCE PUBLISHERS, BARKING, vol. 213, no. 1-2, 15 June 1998 (1998-06-15), pages 159-167, XP004125015 ISSN: 0378-1119 the whole document	1-84
A	ALEXANDER ARONSHTAM ET AL.: "Dominant negative mutator mutations in the mutL gene of Escherichia coli" NUCLEIC ACIDS RESEARCH, vol. 24, no. 13, 1996, pages 2498-2504, XP002168605 cited in the application the whole document	2-5
A	MARILIA CASCALHO ET AL.: "Mismatch repair co-opted by hypermutation" SCIENCE, vol. 279, 20 February 1998 (1998-02-20), pages 1207-1210, XP002168607 DC the whole document	1-84

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/05447

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA 2240609 A		NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)